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Frequent Loss of the Cyclin-dependent Kinase-4 Inhibitor Gene in Human Gliomas

Akira Gomi,[1] Ryuichi Sakai,[2] Seishi Ogawa,[3] Souji Shinoda,[1] Hisamaru Hirai[3] and Toshio Masuzawa[1]

[1]Department of Surgical Neurology, [2]Molecular Biology Division, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-04 and [3]The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113

Studies have shown that homozygous deletion of the cyclin-dependent kinase-4 inhibitor (CDK4I) gene, which is mapped to chromosome 9p21, is frequently observed in various types of human cancers. Here we report that the CDK4I gene was deleted in gliomas. Eight cell lines derived from glioblastomas and samples from 14 patients with various grades of gliomas were examined by Southern blot analysis. We found that the CDK4I gene was deleted in 7 of 8 (87.5%) cell lines and 7 of 9 (78%) samples from high-grade glioma patients, whereas it was deleted in 1 of 5 (20%) low-grade glioma samples. These results suggested that inactivation of the CDK4I gene may play an important role in the progression of human glioma.

Key words: CDK4I — p16 — Glioma

Structural abnormalities involving the short arm of chromosome 9 (9p) are frequently observed in a wide spectrum of human tumors.[1–12] The overlapping region of deletions in these malignancies has been found to be located on 9p21.[3,4,9,12] Previous reports have also detected alterations of 9p21 in gliomas, and moreover, these events were more frequently observed in cell lines derived from malignant gliomas and highly malignant primary tumors as diagnosed histologically, while low-grade gliomas rarely showed deletions.[3,4] Collins and James[13] reported the hypothesis of multistage carcinogenesis of glioma and suggested that alterations of chromosome 9p were related to progression from low-grade glioma (grade I+II) to anaplastic glioma (grade III). Therefore, identification of the gene responsible for tumor progression within the region would be extremely important for understanding the tumor development of gliomas.

Recently, Kamb *et al.* and Nobori *et al.* have proposed that the cyclin-dependent kinase-4 inhibitor (CDK4I) gene is a novel candidate for a tumor suppressor gene.[14,15] CDK4I is a 16 kD inhibitor of the CDK4/cyclin D complexes that control passage through the G1 phase of the cell cycle.[16] The CDK4I gene is localized to 9p21, and has been found to be within the critical deleted region.[14,15,17] To investigate the role of the CDK4I gene in tumorigenesis and the progression of human glioma, we screened the deletion of this gene in samples from patients with various grades of gliomas, as well as in human glioma cell lines, by Southern blot analysis.

We obtained the cell lines analyzed in this study from the Japanese Cancer Research Resources Bank (A172, T98G, U87MG, U251), Dr. Mark Rosenblum (SF188, U138), Dr. Chuji Kasahara (U373), and Dr. Keiji Kawamoto (T430). All of these cell lines were established from tumors diagnosed as glioblastomas (malignancy grade IV). We also examined primary tumors from patients with glioma and menigioma as controls. Tumor specimens were obtained by surgical resection. They were classified morphologically and graded in accordance with the WHO criteria.[18] Gliomas of grade I or II were defined as low grade, while those of grade III or IV were regarded as high grade. Clinical characteristics of the patients, including the biobehavioral grade, are listed in Table I.

DNA was isolated according to the method described previously.[19] Briefly, samples of $1–2\times10^7$ cells or approximately 100 mg of pulverized frozen tumor samples were lyzed in the buffer (containing 0.1 *M* NaCl/0.2 *M* sucrose/0.01 *M* EDTA/0.3 *M* Tris, pH 8.0/0.5% SDS), followed by vortex mixing and incubation at 65°C for 30 min. A 350 μl aliquot of 8 *M* KOAc was added and incubated on ice for 60 min. After centrifugation, the supernatant was extracted with phenol-chloroform and precipitated with ethanol. Purified DNA samples were digested with *Hin*dIII, electrophoresed through 0.7% agarose gels, transferred to nylon membranes (Hybond-N; Amersham), and fixed to membranes by an ultraviolet-activated linker. DNA filters were hybridized with a ^{32}P-labeled CDK4I cDNA probe. The 466bp RT-PCR product of the CDK4I cDNA from a normal bone marrow sample was used as a probe.[20] Autoradiographs of the blots were examined by scanning densitometry, and peak areas corresponding to each hybridization signal were measured by electronic integration. The difference in signals according to amounts of loaded

Table I. Summary of CDK4I Gene Loss in Tumor Samples of Glioma Patients

No.	Age	Sex	Morphology		Grade	Grade of recurrent tumors at initial diagnosis		CDK4I gene
1	62	F	Anaplastic astrocytoma		3			−[a]
2	33	M	Glioblastoma		4			−
3	39	M	Anaplastic astrocytoma	R[d]	3	2		+h[b]
4	73	F	Anaplastic astrocytoma		3			−
5	55	F	Glioblastoma		4			+[c]
6	51	F	Glioblastoma		4			−
7	64	M	Glioblastoma		4			−
8	1	M	Anaplastic astrocytoma		3			+
9	37	M	Anaplastic astrocytoma	R	3	2		−
							high grade gliomas	7/9 (78%)
10	35	F	Ependymoma	R	2	2		+
11	11	M	Ependymoma	R	2	2		−
12	48	M	Oligodendroglioma	R	2	2		+
13	65	M	Ependymoma		2			+
14	15	M	Ependymoma		2			+
							low grade gliomas	1/5 (20%)
							total	8/14 (57%)
15	72	F	Meningioma					+
16	60	F	Meningioma					+
							meningiomas	0/2 (0%)

a) −, Homozygous deletions.
b) +h, Hemizygous deletions.
c) +, Autoradiographic signal of intensity comparable with that of controls. Gene dosage was calculated by normalizing to a control probe (Tec) and using the following calculation: $(CDK4I_T/Tec_T)/(CDK4I_N/Tec_N)\times 2$, where CDK4I represents the signal response for the CDK4I cDNA probe in corresponding normal (N) and tumor (T) DNA samples and Tec represents the signal response at the human Tec cDNA locus on chromosome 4p12. Using this formula, a value of 2.00 would represent a normalized tumor DNA CDK4I signal response equivalent to that produced by the control DNA. The dosage values for CDK4I in the tumors with deletions are as follows: tumor 1, 0.20; 2, 0.43; 3, 1.17; 4, 0.21; 6, 0.48; 7, 0.53; 9, 0.05; 11, 0.59. Dosage values higher than 0 in the tumors with homozygous deletion are due to admixed normal cells.
d) R, recurrent cases.

DNA in gels was calibrated against the signals produced by the control probe. The control probe used was the human Tec cDNA probe located on chromosome 4p12,[21] on which chromosomal abnormalities have rarely been detected in gliomas.[22–24]

We analyzed 8 glioma-derived cell lines (Fig. 1a). Normal CDK4I genomic DNA was detected as three distinct bands of approximately 20 kb, 12 kb, and 6 kb in size. In 7 of 8 cell lines (87.5%), the three bands due to the CDK4I gene could not be detected, although they did appear in the other one. Because the Tec probe could successfully detect its genomic fragment in every lane, it was concluded that the CDK4I gene locus was homozygously deleted in the 7 cell lines. Recently, p15, a novel homologue of CDK4I, was cloned.[25] It may act as an effector of transforming growth factor-beta (TGF-β)-mediated cell cycle arrest. The C-terminal 81 amino acid residues of p15 and CDK4I share 97% homology, which causes cross-hybridization in Southern blot analysis, as we have shown recently. According to our analysis using the p15 specific cDNA probe, the 20 kb band of the three bands was derived from the p15 genomic DNA fragment (unpublished deta).

We have analyzed 16 primary brain tumor samples. Five samples were obtained from recurrent tumors in patients who had undergone radiation and/or chemotherapy treatment following initial surgical resection of the primary tumors, all other specimens were obtained at initial surgery from patients who had not received any treatment. The tumors included 9 high-grade gliomas (4 glioblastomas, 5 anaplastic astrocytomas), 5 low-grade gliomas (4 ependymomas, 1 oligodendroglioma), and 2 menigiomas. We found that in 7 of 9 (77.8%) samples from high-grade glioma patients and in 1 of 5 (20.0%) low-grade glioma samples the normal CDK4I DNA bands were diminished, while the control band was clearly detected (Fig. 1). Although several additional bands were found only in lane 9 of Fig. 1b, they were not detected in

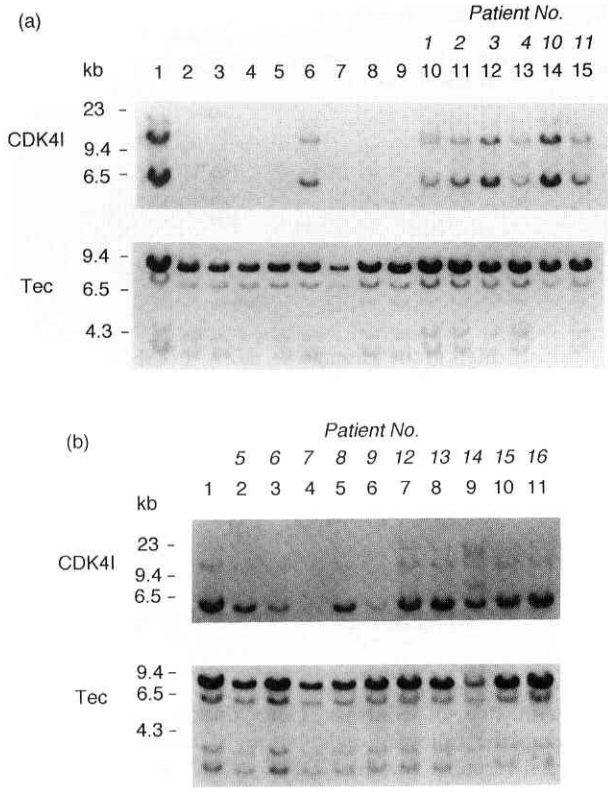

Fig. 1. Southern blot analysis of human glioma cell lines. The CDK4I gene was detected as three discrete bands of approximately 20 kb, 12 kb, and 6 kb in size by *Hin*dIII digestion of genomic DNA. The human Tec gene fragments were rehybridized as an internal control and are shown below. (a) Lane: 1, normal peripheral blood; 2, A172; 3, T98G; 4, U87MG; 5, U251; 6, SF188; 7, U138; 8, T430; 9, U373; from 10 to 13, high-grade glioma samples; 14 and 15, low-grade glioma samples. (b) Lane: 1, normal peripheral blood; from 2 to 6, high-grade glioma samples; 7, 8 and 9, low-grade glioma samples; 10 and 11, meningioma samples.

repeated experiments (data not shown) suggesting that they were probably derived from incompletely digested fragments. There were two recurrent cases that were initially diagnosed as astrocytoma grade II and finally diagnosed as anaplastic astrocytoma grade III (No. 3 and No. 9). In both of these cases, the CDK4I genes were deleted. Deletions were, however, not found in 2 of 3 cases that were diagnosed as grade II gliomas at the initial stage and recurrence. In the last recurrent case, in which the deletion was found, morphological change from ependymoma to a mixture of ependymoma and astrocytoma (grade II) was observed. Deletions of the CDK4I gene were not observed in 2 meningioma samples (Table I).

The cell cycle in eukaryotes is regulated by the cyclin-dependent kinases (CDKs), among which CDK4 is considered to associate with D-type cyclin and to control cell proliferation through the G1 phase. Alteration of the CDK4I gene, therefore, may lead to unregulated proliferation of cells. In this context, recent reports that the CDK4I gene is homozygously lost in a wide variety of human tumor cell lines seem strongly to support the idea that this is the tumor suppressor gene that had been sought for in the 9p21 locus, a frequently deleted chromosomal region in various tumors.[1–17]

We analyzed 14 samples from patients with various grades of gliomas, as well as 8 glioma cell lines, for loss of the CDK4I gene by Southern blot analysis using the probe of the coding region. We found deletions of the CDK4I gene in 87.5% of glioma cell lines, and in 57.1% of samples from glioma patients. These frequencies of deletion in primary glioma samples were extremely high compared with those of other primary tumors previously reported. Ogawa *et al.* have reported that the CDK4I gene was homozygously lost in only 4 of 72 (5.6%) primary leukemia patients,[20] and Spruck *et al.*[26] have detected homozygous deletions in 6 of 31 (19%) samples from primary bladder tumors. With regard to primary tumors, therefore, the frequency of deletions that we found in gliomas was surprisingly high. Kamb *et al.*[14] detected deletions of the CDK4I gene in more than 70% of samples from primary gliomas and glioma-derived cell lines. They, however, detected deletions of sequence-tagged sites (STSs) located in or around the CDK4I gene with polymerase chain reaction (PCR), but these were not necessarily the same as those of the coding region of the CDK4I gene. They did not clearly describe the frequency of deletion in primary tumors. Nobori *et al.*[15] have reported that the frequency of homozygous loss of CDK4I in gliomas was 87.5% as determined with PCR. The frequency is similar to that in our study. They, however, did not examine primary tumor samples. In our study, the frequency of deletion of the coding region was examined in primary glioma samples.

All of the cell lines we examined were established from tumors diagnosed as glioblastomas (malignancy grade IV). In addition, we found CDK4I gene deletions at an extremely high frequency (77.8%) in tumor samples from patients with high-grade gliomas. In brief, the CDK4I gene was frequently deleted in high-grade gliomas and rarely in low-grade gliomas. Moreover, we found deletions of the CDK4I gene in both of the samples obtained from patients with recurrent anaplastic astrocytomas that were initially diagnosed as astrocytoma grade II, whereas deletions were not found in two samples from patients who were diagnosed as having grade II gliomas at recurrence and in the initial stage. These data suggest that deletion of the CDK4I gene may

occur as an event of tumor progression rather than initiation and that the CDK4I gene is the putative gene responsible for tumor progression of gliomas located in 9p21.

Recent reports have indicated that CDK4I gene alterations were more frequently found in cell lines than in primary tumors, and it was suggested that tumor cells with the CDK4I homozygous loss may have a selective growth advantage in tissue culture.[26,27] Spruck *et al.*[26] reported that the frequency of the CDK4I alteration was 19% in primary bladder tumors against 54% in cell lines, and Cairns *et al.*[27] observed the CDK4I mutations in only two of 75 various primary tumors. We, however, found CDK4I alterations in 78% of tumor samples of malignant gliomas, and Mori *et al.*[28] found somatic mutations of the CDK4I gene in 14 of 27 (52%) esophageal squamous cell carcinomas. These data suggest that the CDK4I gene plays an important role not only in the establishment of cell lines, but also in tumorigenesis. The CDK4I gene may be important in some types of tumors but not in others. In this study we did not examine other mechanisms through which the CDK4I gene might be inactivated, for example, transcriptional or translational inhibition and point mutation. These possibilities should be addressed in glioma patients in further studies.

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